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CYTOLOGICAL AND IMMUNOLOGICAL INVESTIGATIONS OF SPERM–EGG INTERACTIONS IN SELECTED DECAPODS (CRUSTACEA) AND *LIMULUS POLYPHEMUS* L. (MEROSTOMATA)[1]

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The physiological aspects of sperm-egg interactions have been studied extensively by many investigators (see Metz and Monroy, 1967 for review). These studies include such topics as the effects of egg substances on specificity of attachment, activation of the sperm acrosomal reaction, and release of lytic substances from the acrosome. Many, if not all, of these processes evidently involve gamete surface components; *e.g.*, macromolecules on the surface of the gametes which are believed to interact much like enzyme and substrate or antigen and antibody. Most of this work has been done on echinoderm and amphibian gametes (Metz, 1967 for review).

The present study deals with gamete specificity and the role of gamete surface components, especially antigens, in the fertilization of several representative species of decapod crustaceans and a xiphosuran, *Limulus polyphemus*. The morphology of decapod spermatozoa is quite unusual and bizarre (Brown, 1966) as compared to the so-called typical spermatozoa (*e.g.*, sea urchin sperm; Franklin, 1965). The reptantian spermatozoa used in this study are large and non-motile, possess a number of radiating arms, and have a very large and complex acrosome. Initial sperm-egg attachment is polyspermic. These spermatozoa need to be studied physiologically because their interactions with the egg during fertilization should be compared with those of the typical sperm, upon which most previous physiological information is based. In addition, the large size of the decapod sperm facilitates the analysis of its surface components and their behavior during sperm-egg interactions. Finally, since these spermatozoa are non-motile, one of the usual parameters in fertilization, namely sperm motility, is eliminated and the system is correspondingly simplified.

Materials and Methods

Live specimens were obtained from three sources: (1) the Marine Biological Laboratory Supply Department, Woods Hole, Massachusetts, (2) the Gulf Speci-

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men Company, Panacea, Florida, and (3) the Florida Marine Biological Specimen Company, Panama City, Florida.

Gametes were obtained from decapods by dissection. Free spermatozoa were obtained from the testes and the seminal receptacle of the female. Spermatozoa contained in spermatophores were obtained from the vas deferens. Mature eggs and oocytes were obtained by maceration of ovarian tissue. Oocytes were considered adequate for experimental use if spermatozoa attached to them in normal proportions (*i.e.*, comparable to the number of spermatozoa attached to normal eggs). Spermatozoa and eggs were obtained from *Limulus polyphemus* males and females by electrical stimulation (Shrank, Shoger, Schechtman and Bishop, 1967).

Antisera were prepared by injecting rabbits subscapularly with an emulsion of

TABLE I

*Types of antisera prepared, the species of animals used and their source**

Antiserum	Antigen prepared from	Species	Source of animals
Anti-*Callinectes* spermatophores	sperm filled spermatophores	*Callinectes sapidus* Rathbun	Gulf Specimen Co.
Anti-*Callinectes* sperm	seminal receptacle sperm	*Callinectes sapidus*	Flor. Mar. Biol. Spec. Co.
Anti-*Callinectes* eggs	eggs	*Callinectes sapidus*	Gulf Specimen Co.
Anti-*Libinia* spermatophores	sperm filled spermatophores	*Libinia emarginata* Leach	Marine Biol. Lab.
Anti-*Pagurus* spermatophores	sperm filled spermatophores	*Pagurus pollicaris* (Say)	Marine Biol. Lab.
Anti-*Homarus* vas deferens	vas deferens	*Homarus americanus* Milne-Edwards	Marine Biol. Lab.
Anti-*Limulus* sperm	sperm	*Limulus polyphemus* Linnaeus	Marine Biol. Lab.

* Other species used in this study (*Cancer irroratus* Say and *Ovalipes ocellatus* (Herbst) were obtained from the Marine Biological Laboratory.

whole intact sperm and Freund's complete adjuvant (1:3). Table I presents the kinds of antisera prepared, the species used, and the source of the animals. To assure that equivalent amounts of antibody were used in all experiments, the globulin fraction was separated from the whole serum by precipitating with 18% sodium sulfate (Kekwick, 1940). Protein determinations were made on the globulin fractions using the biuret method (Gornall, Bardawill and David, 1949) and the antibody solutions were then adjusted to equivalent concentrations (25 mg protein per milliliter).

Extracts of sperm and eggs for immunodiffusion experiments were made by homogenizing gametes in a glass homogenizer at 0° C in sea water, 0.85% saline or 0.8 *M* Tris buffer at pH 7.0. In some cases the gametes were treated with 0.5% sodium deoxycholate to enhance membrane disruption. After homogenization the suspension was freeze thawed in an acetone-dry ice bath to aid in the release of antigens from the membranes. In some cases the suspensions were also treated ultrasonically to break up the cell membranes. This consisted of four, 15 second bursts on a Branson sonifier at 0° C. Cellular debris was removed by centrifuga-

tion. In most cases the extracts were treated with 0.05% deoxyribonuclease to digest any DNA present which may bind with proteins and precipitate them. Extracts were made up to a ratio of 1:6 tissue to buffer. Antibody concentration was 25 mg protein per milliliter. The gel was prepared from 1% agarose in 0.85% saline with 0.2% sodium azide added as a preservative and 0.05% cadmium chloride added to enhance antigen-antibody precipitate formation.

Some experiments employed papain-digested antibody prepared by the method of Porter (1959) under the following conditions: 100 mg globulin/mg papain (2X crystallized, Sigma Chemical Co.) in 0.1 *M* potassium phosphate, 0.01 *M* cystein, 0.002 *M* disodium EDTA; pH 7.0, 37° C. Digestion was considered complete (usually after 18–22 hours) when the antibody no longer agglutinated

TABLE II

Results of cross-insemination experiment reported as the average number of spermatozoa attached per millimeter egg circumference

Spermatozoa	Eggs					
	Libinia	*Callinectes*	*Cancer*	*Ovalipes*	*Homarus*	*Limulus*
Libinia	12.5±3.4*	0.3±0.15	0	0	0	0
Callinectes	0.4±0.22	14.1±3.5	0	0	0	0
Cancer	0	0	43.2±6.5	0	0	0
Ovalipes	0	0	0	8.0±2.2	0	0
Homarus	0	12.8±2.7	9.6±3.3	6.0±1.7	90.6±10.1	0
Limulus	0	0	0	0	0	75.8±9.2

* Standard deviation.

Washed spermatozoa were made up to a 2% suspension. From 10–30 eggs were placed in the well of a spot plate and the excess water removed. Two drops of 2% sperm suspension were added to the well and this mixture was stirred for one minute and then the eggs were washed three times with an excess of sea water. The eggs were then transferred to a microscope slide and observed with phase contrast optics. Ten eggs were chosen randomly and the number of spermatozoa attached to each was determined and recorded. The eggs were not rotated; only the sperm around the egg periphery were counted. The experiment was set up all at one time so that all eggs were treated with the same sperm suspension of each species, *etc.*

homologous sperm in a 2% suspension. At this time iodoacetamide was added to a final concentration of 0.02 *M* to inactivate the enzyme. The digests were then dialyzed exhaustively against sea water.

Fluorescein-conjugated antibody was prepared by the method of Riggs, Seiwald, Burchhalter, Downs and Metcalf (1958). Conditions for this procedure include 0.15 *M* NaCl, 8% acetone, 0.06 *M* carbonate-bicarbonate buffer (pH 9.0), 10 mg globulin per milliliter, and 0.05 mg fluorescein isothiocyanate (Nutritional Biochemicals Corp., Cleveland, Ohio) per milligram globulin. This mixture was stirred at 4° C for 18 hours. The conjugated protein fraction was isolated by column chromatography (16 × 2.5 cm column) with 0.1 *M* phosphate buffer (pH 7.2) in Sephadex G-25 med. and then dialyzed against sea water for 72 hours. Sperm suspensions (2%) were treated with fluorescein-conjugated antibody for 5 minutes and then washed with sea water. These suspensions were then observed with a Zeiss fluorescence microscope.

Results

Sperm-egg attachment specificity

Five different types of experiments were performed on decapod and *Limulus* gametes. These included cross-insemination, cross-absorption-agglutination, immunodiffusion, antibody inhibition, and antigen localization experiments. These experiments were designed to test for the following: (1) the presence of molecular components essential for fertilization on the surface of sperm and egg, (2) the species specificity of such components, (3) the involvement of such components

Table III

Results of cross-absorption-agglutination experiments

Sera		Spermatozoa						
Prepared against sperm	Absorbed with sperm	*Libinia*	*Pagurus*	*Homarus*	*Limulus*	*Ovalipes*	*Cancer*	*Callinectes*
Control serum		0	0	0	0	0	0	0
Libinia	—	+++	0	+	0	0	0	—
	Homarus	+++	—	0	—	—	—	
Pagurus	—	0	+++	++	0	0	0	—
	Homarus	—	0	0	—	—	—	
Homarus	—	0	+	++	0	0	0	—
	Pagurus	—	0	0	—	—	—	
Limulus	—	0	+	0	+++	0	+	—
	Pagurus	—	0	—	++	—	+	
	Cancer	—	0	—	++	—	0	
Callinectes		+	0	+	0	0	+	+++

+ degree of agglutination; 0 = no agglutination; — = no test performed.

Agglutinations were performed with anti-sperm globulin previously prepared against the sperm of each species. The globulin solutions were all diluted to a concentration of 1.6 mg protein per ml, as this was the concentration at which control globulin no longer agglutinated any of the sperm types. One drop of a 2% sperm suspension was mixed together with one drop of anti-sperm globulin on a microscope slide. All tests included two controls: one treated with sea water and one treated with control globulin. Absorptions were performed by adding whole sperm to the globulin and allowing it to stand for three hours at 5° C.

in sperm-egg attachment, (4) the antigenicity of the components, and (5) the localization of antigenic components with labeled antibody.

Reciprocal sperm-egg crosses were performed *in vitro* between the following species: *Libinia emarginata, Callinectes sapidus, Ovalipes ocellatus, Cancer irroratus, Homarus americanus*, and *Limulus polyphemus* (Table II). Among the crustaceans employed in these experiments, sperm-egg attachment is polyspermic and most if not all of the attached spermatozoa remain visible at the egg surface for several minutes. Accordingly, comparison of the numbers of spermatozoa attached to the egg surface should provide a measure of sperm-egg affinity. The results of the experiments support this view. Two preliminary qualitative experiments were performed (results not reported). The results of a third experiment are given in Table II as the average number of spermatozoa attached per egg.

All three experiments agree. The results show that with one exception the attachment of gametes of all the species represented are highly self-specific. The spermatozoa of each species attach only to their own eggs. In the one exceptional case *Homarus* spermatozoa attach to the eggs of three other species, *Callinectes*, *Cancer* and *Ovalipes*. These attachments were less firm than normal and the acrosome often was oriented away from the egg surface. *Homarus* eggs failed to cross-attach to the spermatozoa of these three species.

Antigenic specificity

Antibodies were used in cross-absorption-agglutination tests on spermatozoa from the following species: *Libinia emarginata, Callinectes sapidus, Cancer irroratus,*

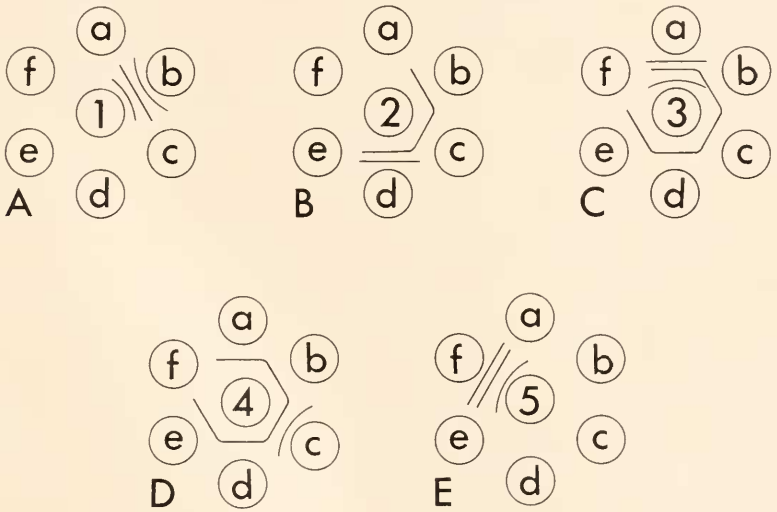

FIGURE 1. Immunodiffusion tests; (1) anti-*Homarus* sperm, (2) anti-*Pagurus* sperm, (3) anti-*Callinectes* sperm (4) anti-*Libinia* sperm, (5) anti-*Limulus* sperm, (a) Callinectes sperm extract, (b) *Homarus* sperm extract, (c) *Libinia* sperm extract, (d) *Pagurus* sperm extract, (e) *Cancer* sperm extract, (f) *Limulus* sperm extract.

Homarus americanus, Pagurus pollicaris, and *Limulus polyphemus* (Table III). Three sets of experiments were performed. All antibodies readily agglutinated their own spermatozoa but none of the antibodies agglutinated the spermatozoa of every species. The agglutinations indicate that antigens are on the surfaces of the spermatozoa. Antigens are present over the entire surface since arm to arm, arm to "head" and "head" to "head" agglutinations occurred. The arm to "head" agglutinations mean that the same antigen appears on the arm and "head." The possibility of arm and "head" specific antigens has not been excluded. The experiments indicate that *Libinia* sperm has at least two surface antigens, one of which is shared with *Homarus* sperm. *Homarus* sperm has at least one antigen which it shares with *Pagurus* sperm. *Limulus* sperm has at least three surface antigens, sharing one of them with *Pagurus* sperm and another with *Cancer* sperm.

This test also indicates that *Pagurus* sperm shares an antigen with *Cancer* sperm. Anti-*Callinectes* sperm serum agglutinates *Libinia*, *Homarus* and *Cancer* spermatozoa. In synopsis, the spermatozoa of *Homarus* has at least one and *Libinia* and *Pagurus* has at least two surface antigens. The sperm of *Limulus* has at least three surface antigens.

Soluble antigens

Immunodiffusion tests (Fig. 1) were performed on extracts of the same species of spermatozoa as used in cross-absorption–agglutination experiments. The extracted antigens could originate from the surface, the interior or both parts of the spermatozoan. A specific antibody was placed in the center well and sperm

TABLE IV

Results of inhibition experiments reported as the average number of sperm attached per millimeter of egg circumference

Experiment	Untreated	*Libinia emarginata* spermatozoa treated with:				
		Undigested anti-*Libinia* sperm serum	Digested anti-*Libinia* sperm serum	Undigested control serum	Digested control serum	Digested anti-*Libinia* sperm serum and sheep anti-rabbit globulin serum
1	13.6 ± 3.1	0	0.1 ± 0.10	4.3 ± 1.8	16.3 ± 4.5	—
2	14.9 ± 3.7	0	0.4 ± 0.15	7.9 ± 2.9	5.1 ± 2.1	0.2 ± 0.12

Two drops of a 2% sperm suspension was mixed together with 1 drop of globulin (25 mg protein per ml) in the well of a spot plate. This mixture was agitated for five minutes and then eggs (10–30) were added. After two minutes the excess sperm were washed away and the eggs were examined to determine the numbers of sperm attached. Ten eggs were counted.

extracts in the peripheral wells. Precipitin band formation revealed that each species has at least two soluble antigens. *Pagurus* (Fig. 1B), and *Libinia* (Fig. 1D), spermatozoa each have only two antigens. *Homarus* (Fig. 1A), *Limulus* (Fig. 1E) and *Callinectes* (Fig. 1C) spermatozoa have three antigens. The reaction with anti-*Pagurus* sperm globulin indicates that *Pagurus* sperm shares one common antigen with *Homarus* and *Libinia* spermatozoa (Fig. 1B). Likewise, *Callinectes* sperm shares one common antigen with *Homarus, Libinia, Pagurus,* and *Cancer* spermatozoa (Fig. 1C), and *Libinia* sperm shares one common antigen with *Callinectes, Homarus, Pagurus,* and *Cancer* spermatozoa (Fig. 1D).

Evidence for functional sperm antigens in attachment

Sperm surface antigens can be blocked with specific antibodies to test for their possible role in fertilization. In these experiments *Libinia* spermatozoa were treated with bivalent or univalent anti-sperm globulin (Table IV). The treated spermatozoa were then mixed with eggs in order to determine their capacity

for attachment. Bivalent anti-sperm globulin treatment reduced the attachment capacity of the sperm. This result was probably influenced by the fact that whole antibody agglutinates the sperm into large clumps which effectively prevents them from interacting with the egg. However, spermatozoa treated with univalent, non agglutinating anti-sperm globulin also failed to attach to the eggs. Spermatozoa pretreated with digested anti-sperm globulin agglutinate upon subsequent treatment with sheep anti-rabbit globulin (Coombs' or anti-globulin test, Coombs', Mourant and Race, 1945). Treatment of spermatozoa with both whole and digested control globulin did not markedly reduce their ability to attach to the egg.

Localization of sperm antigens by immunofluorescence

Libinia spermatozoa were treated with fluorescein-labeled anti-*Libinia* sperm globulin. Fluorescence was observed over the entire surface of each sperm. No specific area fluoresced more than another. Non-specific staining was checked by treating spermatozoa with fluorescein-labeled control globulin. Such control treated spermatozoa did not fluoresce.

Discussion

The large, non-motile spermatozoa of decapod crustacea should be unusually favorable for studying the initial stages of sperm-egg attachment and interaction. The present study supports this view. It demonstrates species specificity of sperm-egg attachment, specific sperm surface antigens and, at least in *Libinia,* apparent involvement of sperm surface antigens in sperm-egg attachment. Thus the decapods resemble the sea urchin and the few other forms (Tyler, 1946; Metz, Schuel and Bischoff, 1964) that have been studied. The present study extends the analysis to the extent that one well defined process, namely sperm-egg attachment, is inhibited by antibody. No clear cut evidence is yet available to specify the fertilization step or steps inhibited by antisperm sera in other metazoa (*e.g.,* Metz, 1967). It may be argued that the conditions of *in vitro* insemination employed here are sufficiently abnormal to produce excessive and pathological polyspermic sperm-egg interaction. However, Hinsch (in preparation) has examined naturally fertilized eggs of *Libinia* shortly after spawning and finds that these also are highly polyspermic. It will now be of particular interest to determine if the antibody treated spermatozoa can undergo the acrosomal reaction, in other words if the attachment block is at the pre or post acrosomal reaction stage. In the sea urchin the acrosomal reaction is not affected by antibody pretreatment (Fourtner and Metz, 1967).

The cross-absorption agglutination and precipitin tests show that the crustacean spermatozoa have constellations of surface antigens. The distribution and number of these antigens and their solubility can now be extended using additional interspecific combinations and appropriate absorbed sera. Additionally, it may be possible to identify and characterize the "attachment" antigen, using specific antibody as a label. Finally, the interesting question of the antigenic relationship between the surface of the reacted acrosome and the rest of the spermatozoa can probably be investigated readily in this material.

Summary

The initial events of sperm-egg interaction have been examined in several crustaceans. These have large non-motile spermatozoa and sperm-egg attachment is polyspermic.

Sperm-egg attachment *in vitro* is largely species specific among the organisms examined.

Antisperm sera produced in rabbits agglutinates the crustacean spermatozoa. Interspecific agglutinations do occur. Appropriate absorption experiments demonstrate more than one sperm surface antigen.

Spermatozoa pretreated with univalent, non-agglutinating antibody fail to attach to eggs of the species. This indicates that one or more sperm surface antigens are involved in sperm-egg attachment.

Antigens are distributed over the entire sperm surface as shown by the morphology of agglutination and by immunofluorescence.

Two or more soluble antigens can be extracted from crustacean spermatozoa, depending upon species. Some of these antigens give interspecific reactions, others are species specific.

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